Effect of saponin treatment on the sarcoplasmic reticulum of rat, cane toad and crustacean (yabby) skeletal muscle

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1. Mechanically skinned fibres from skeletal muscles of the rat, toad and yabby were used to investigate the effect of saponin treatment on sarcoplasmic reticulum (SR) Ca^{2+} loading properties. The SR was loaded submaximally under control conditions before and after treatment with saponin and SR Ca^{2+} was released with caffeine.

2. Treatment with 10 $\mu g\ ml^{-1}$ saponin greatly reduced the SR Ca^{2+} loading ability of skinned fibres from the extensor digitorum longus muscle of the rat with a rate constant of 0·24 min^{-1}. Saponin concentrations up to 150 $\mu g\ ml^{-1}$ and increased exposure time up to 30 min did not further reduce the SR Ca^{2+} loading ability of the SR, which indicates that the inhibitory action of 10–150 $\mu g\ ml^{-1}$ saponin is not dose dependent. The effect of saponin was also not dependent on the state of polarization of the transverse-tubular system.

3. Treatment with saponin at concentrations up to 100 $\mu g\ ml^{-1}$ for 30 min did not affect the Ca^{2+} loading ability of SR in skinned skeletal muscle fibres from the twitch portion of the toad iliofibularis muscle but SR Ca^{2+} loading ability decreased markedly with a time constant of 0·22 min^{-1} in the presence of 150 $\mu g\ ml^{-1}$ saponin.

4. The saponin dependent increase in permeability could be reversed in both rat and toad fibres by short treatment with 6 μM Ruthenium Red, a potent SR Ca^{2+} channel blocker, suggesting that saponin does affect the SR Ca^{2+} channel properties in mammalian and anuran skeletal muscle.

5. Treatment of skinned fibres of long sarcomere length (> 6 μm) from the claw muscle of the yabby (a freshwater decapod crustacean) with 10 $\mu g\ ml^{-1}$ saponin for 30 min abolished the ability of the SR to load Ca^{2+}, indicating that saponin affects differently the SR from skeletal muscles of mammals, anurans and crustaceans.

6. It is concluded that at relatively low concentrations, saponin causes inhibition of the skeletal SR Ca^{2+} loading ability in a species dependent manner, probably by increasing the Ca^{2+} loss through SR Ca^{2+} release channels.

Saponin is an agent known to interact with cholesterol molecules in the sarcolemma and the transverse-tubular system (t-system) of muscle fibres causing perforations in the membranes (Endo & Kitazawa, 1978). The perforation of the cellular membrane with saponin allows ready access to the intracellular membrane systems, which contain little cholesterol (Meissner & Fleischer, 1971) and therefore are believed not to be affected by saponin treatment. Chemical skinning with saponin has become widely used to study properties of the sarcoplasmic reticulum (SR) in cardiac, smooth and skeletal muscle. Endo & Iino (1980) have shown that saponin at 100 $\mu g\ ml^{-1}$ or less does not significantly affect the Ca^{2+} loading properties of the SR in skeletal muscle from African clawed toads (*Xenopus* sp.); however, Herland, Julian & Stephenson (1990) have reported that exposure of mammalian ventricular preparations to 50 $\mu g\ ml^{-1}$ saponin for longer than 10 min impaired the SR ability to accumulate Ca^{2+}. This raises the possibility that saponin may affect the SR function differently in different muscle types from different animals. At present there is no comprehensive study of the effect of saponin on the SR properties of skeletal muscle fibres from different animals.

A suitable preparation for studying the effect of saponin on SR Ca^{2+} loading properties is the mechanically skinned fibre where the surface membrane is removed by microdissection and where the myoplasmic environment is open to experimental manipulation while retaining normal structure and functioning of the SR (Endo, 1977; Fink & Stephenson, 1987). Therefore, it is possible to examine the Ca^{2+} loading properties of the SR before and after saponin treatment.

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The aim of this study was to determine if the SR Ca^{2+} loading ability is affected by saponin treatment in mechanically skinned skeletal muscle fibres from three major groups of animals in which muscle function is intensely studied: anurans, mammals and crustaceans. Some of the results have been published in abstract form (Launikonis & Stephenson, 1996).

METHODS

Animals and solutions

Male rats (Long Evans, hooded; 200–300 g, 3 months old) were killed by asphyxiation under deep anaesthesia with halothane (2% v/v). The extensor digitorum longus (EDL) muscles were dissected, well blotted on filter paper (Whatman No.1) and then placed in a Petri dish containing paraffin oil (Ajax Chemicals, Sydney, Australia) above a layer of Sylgard 184 (Dow Chemicals, Midland, MI, USA). Cane toads (*Bufo marinus*) were stunned with a heavy blow to the head and then killed by double pithing. The iliofibularis muscles were removed, well blotted on filter paper and placed in a Petri dish on a layer of Sylgard under paraffin oil. Single muscle fibres were then isolated and mechanically skinned with fine forceps under paraffin oil. Either the left or right chelae of the freshwater decapod crustacean *Cherax destructor* (yabby; Central Victorian Yabby Farm, South Heathcote, Victoria, Australia) were removed and placed in a bathing solution containing (mM): NaCl, 207; KCl, 5·4; $CaCl_2$, 13·6; $MgCl_2$, 2·64; and Pipes, 3 (pH 7·4 with NaOH). Several incisions were made into the surface of the chelae to expose the muscle fibres. The large apodeme was cut so that intact fibres remained attached between this cartilage and the exoskeleton. Individual fibres were cut free, blotted on filter paper, placed in a Petri dish under paraffin oil above a layer of Sylgard and then mechanically skinned while under the paraffin oil. All yabby fibres used in experiments were of long sarcomere type (resting sarcomere length, > 6 μm). All vertebrates were killed under permits granted by the Animal Ethics and Experimentation Committee (Biological Sciences) at La Trobe University. The mechanically skinned fibre segments from all animals were attached between a pair of platform-ended forceps and a force transducer (AME875, SensoNor 801, Horten, Norway) whilst under oil and the length (L) and diameter (D) were measured using a dissecting microscope as described previously (Lamb & Stephenson, 1990*a*). The attached fibre was then stretched by 20% to increase the sensitivity for detection of Ca^{2+} release in any part of the skinned fibre preparation.

Fibres were then bathed for 2 min in 'high-K^+ solutions' mimicking the myoplasmic environment. These solutions contained (mM): K^+, 117 (toad) or 126 (rat and yabby); Na^+, 36; free Mg^{2+}, 1; hexamethylene-diamine-tetraacetic acid (HDTA; Fluka, Buchs, Switzerland), 50; total ATP, 8; phosphocreatine, 10; NaN_3, 1; EGTA, 0·5 (unless otherwise stated; $[Ca^{2+}] < 10$ nM); sucrose, 0 (rat and toad) or 90 (yabby); and Hepes, 90 (rat and yabby) or 60 (toad); pH 7·10 ± 0·01. All experiments were conducted at 22 ± 2 °C. Skinned fibres were loaded with Ca^{2+} in 'loading solutions' which were matched to the high-K^+ solutions except that they contained 50 μM EGTA and a free $[Ca^{2+}]$ of 0·2 μM (measured with a Ca^{2+}-sensitive electrode; Orion Research, Cambridge, MA, USA). SR Ca^{2+} in loaded skinned fibres was released in solutions containing 30 mM caffeine (added as solid) after being pre-equilibrated for 30 s (unless otherwise stated) in high-K^+ solution. These 'Ca^{2+} release solutions' were similar in composition to the high-K^+ solutions for toad and yabby except for the presence of caffeine; the free $[Mg^{2+}]$ of the Ca^{2+} release solution for rat skinned fibres was reduced to 50 μM to facilitate the opening of the Ca^{2+} release channel (Lamb & Stephenson, 1991*a*; Fryer & Stephenson, 1996) and like the other Ca^{2+} release solutions contained 0·5 mM EGTA. Ruthenium Red (RR; 6 or 50 μM) and saponin (10–150 μg ml^{-1}) were added to high-K^+ solutions from stock solutions of 1·2 mM in distilled water and 2 mg ml^{-1} in high-K^+ solutions, respectively. Solutions containing Triton X-100 were similar in composition to high-K^+ solutions except that EGTA was reduced from 0·5 to 0·1 mM and Triton X-100 was present at 3% v/v. Maximum Ca^{2+} activation was achieved in 'maximum Ca^{2+}-activation solutions' which were similar to the high-K^+ solutions but 50 mM HDTA was replaced with 50 mM CaEGTA/EGTA (20 μM Ca^{2+}) and total [magnesium] adjusted to maintain 1 mM free Mg^{2+}. Fibres were relaxed in 'high-K^+ relaxing solutions' which contained 50 mM EGTA instead of HDTA ($[Ca^{2+}] < 1$ nM). All solutions were prepared according to the methods described by Stephenson & Williams (1981). The osmolalities of all rat, toad and yabby solutions were 290 ± 10, 250 ± 10 and 380 ± 10 mosmol kg^{-1}, respectively. In some experiments with rat muscle fibres K^+ was replaced with Na^+ in all solutions.

Experimental protocols

Caffeine-induced force responses can be used to estimate the relative amount of Ca^{2+} in the SR (Endo & Iino, 1980) by referring to relative areas under the caffeine-induced force responses (see Fig. 2). The pCa ($-\log_{10}[Ca^{2+}]$) of the loading solutions was 6·7. After skinning and equilibration in the appropriate high-K^+ solution for 2 min, the endogenous SR Ca^{2+} in the skinned fibres was released with caffeine in the corresponding Ca^{2+} release solution. The fibres were left in the Ca^{2+} release solution for 2 min to empty thoroughly the SR of Ca^{2+} (Fryer & Stephenson, 1996). Skinned fibres were normally loaded for 2 min in the loading solution and were then placed in high-K^+ solutions for 30 s before transfer to the respective Ca^{2+} release solution. Force was continuously recorded on a chart recorder (linear) and the relative area under the caffeine-induced force response was measured using a gravimetric method (Fink & Stephenson, 1987). The amount of Ca^{2+} loaded in the SR under our conditions was similar to endogenous Ca^{2+} in the SR as judged from the caffeine-induced force response immediately after skinning the preparation. Thus, skinned fibres were loaded submaximally with Ca^{2+} under conditions close to those occurring *in vivo*.

Effect of saponin treatment on SR Ca^{2+} loading ability

The effect of saponin treatment on the SR ability to load Ca^{2+} was investigated by loading the SR submaximally as stated above and releasing the SR Ca^{2+} with 30 mM caffeine in Ca^{2+} release solution after 30 s pre-equilibration in high-K^+ solution. Loading conditions were kept constant throughout all experiments and produced caffeine-induced force responses that were between 30 and 60% maximum Ca^{2+}-activated force (max force), which was always measured at the end of an experiment (see Fig. 2). After three cycles in which Ca^{2+} was loaded in the SR and subsequently released with caffeine (to verify reproducibility), fibres were treated with saponin (10–150 μg ml^{-1}) in high-K^+ solution for 30 min (unless otherwise stated). After saponin treatment, fibres were thoroughly washed in high-K^+ solution and then Ca^{2+} was loaded and released under the same conditions as before the saponin treatment. The area under the caffeine-induced responses after exposure to high-K^+ solution containing different concentrations of saponin was expressed as a fraction of the area before exposure to saponin in the same preparation for the same Ca^{2+} loading conditions. The effect of saponin treatment on SR Ca^{2+} loading ability was assessed by comparing the relative area calculated as

indicated above with the relative area from control experiments where the preparations were incubated in high-K^+ solution without saponin for the same period of time. Paired caffeine-induced force responses were normally obtained before and after exposure to high-K^+ solution with and without saponin (Figs 2, 4 and 6) and the average of the areas under these paired responses was used to calculate the relative responses (Figs 3*A* and 5*A*).

Effect of saponin treatment on contractile apparatus

To determine if saponin treatment induced changes in Ca^{2+} sensitivity of the contractile apparatus, skinned fibres were activated submaximally in a solution of pCa 6·0 which was prepared from a combination of maximum Ca^{2+}-activation solution and relaxing solution and then maximally activated at pCa 4·5 in maximum activation solution. Fibres were exposed to these activating solutions before and after a 30 min period with 10 (yabby), 100 (rat) or 150 μg ml^{-1} saponin (toad) in high-K^+ solution. Relative proportions of submaximal to maximal Ca^{2+}-activated force responses before and after 30 min treatment with saponin were compared to assess the effect of saponin treatment on the Ca^{2+} sensitivity of the contractile apparatus.

SR Ca^{2+} leak experiments

Assuming that the rate of Ca^{2+} loss from the SR is proportional to the permeability for Ca^{2+} (P_{Ca}) and to the $[Ca^{2+}]$ in the SR ($[Ca^{2+}]_{SR}$), i.e.

$$d[Ca^{2+}]_{SR}/dt = -\alpha P_{Ca}[Ca^{2+}]_{SR}, \quad (1)$$

where α is a proportionality constant, it follows that the Ca^{2+} content of the SR would decrease exponentially if P_{Ca} remained constant,

$$[Ca^{2+}]_{SR} = [Ca^{2+}]_{SR,0} \exp(-\alpha P_{Ca} t), \quad (2)$$

where $[Ca^{2+}]_{SR,0}$ is the amount of Ca^{2+} in the SR at time 0.

If SR Ca^{2+} is allowed to leak out for two time intervals, t_1 and t_2, starting from the same initial $[Ca^{2+}]_{SR,0}$, then the relative amount of Ca^{2+} left in the SR after t_1 and t_2 will be given by:

$$[Ca^{2+}]_{SR,t_1}/[Ca^{2+}]_{SR,t_2} = \exp(-\alpha P_{Ca}(t_1 - t_2)). \quad (3)$$

If t_1 and t_2 are kept constant, the ratio $[Ca^{2+}]_{SR,t_1}/[Ca^{2+}]_{SR,t_2}$, defined as the Ca^{2+} retention index, gives a direct indication of the permeability of the SR membrane to Ca^{2+}.

In our experiments we have chosen $t_1 = 120$ s and $t_2 = 30$ s and from the respective areas under the caffeine-induced force responses, we estimated $[Ca^{2+}]_{SR,120s}/[Ca^{2+}]_{SR,30s}$ under control conditions and after 1 min exposure to 6 μM RR, both before and after exposure to saponin treatments. The preparations were always loaded with Ca^{2+} to the same initial level for each set of leak measurements comprising three caffeine-induced force responses after 30 s, 120 s and again 30 s exposure to high-K^+ solution immediately before exposure to the Ca^{2+}-release solution. The Ca^{2+} retention index was calculated as the ratio between the area under the 120 s leak response and the mean areas under the 30 s leak responses before and after (see Fig. 8). Treatment with RR was found to slow the rate of rise and increase the area of the caffeine-induced force responses compared with caffeine-induced responses in the absence of RR (data not shown). Since a change in sensitivity to Ca^{2+} of the contractile apparatus and/or the kinetics of the caffeine-induced Ca^{2+} release may change the area under the force response for the same amount of SR Ca^{2+} released, it is more appropriate for comparative purposes to use the Ca^{2+} retention ratio calculated for each treatment of the preparation rather than the area under the caffeine-induced responses before and after such treatment.

Estimation of relative SR Ca^{2+} content for the leak experiments

The areas under the appropriate caffeine-induced force responses could be used to estimate the Ca^{2+} retention index for particular conditions providing that the area and the SR Ca^{2+} content are directly proportional to each other. Figure 1 shows results obtained with twenty mechanically skinned rat fibres loaded for different time intervals in the loading solution. In order to facilitate the analysis of data points from different fibres, the area data points for individual fibres have been divided by the area obtained under standard loading conditions (120 s) for that particular fibre. The results in Fig. 1 show that for our conditions there is a tight linear dependence of the area under the caffeine-induced force response and the loading time. Since the amount of Ca^{2+} loading in the SR is expected to increase linearly with time under our conditions, it

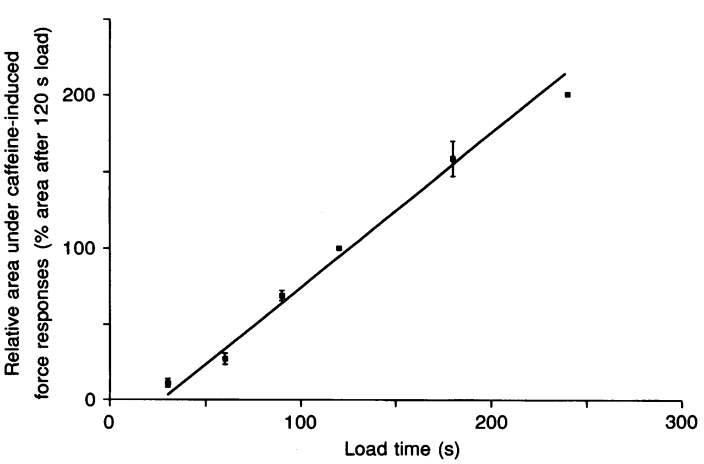

Figure 1. The relationship between mean areas under the caffeine-induced force responses (see Fig. 2) and load time in the loading solution obtained with 20 mechanically skinned rat fibres

The data points for the individual fibres were normalized to the area obtained under standard loading conditions (120 s) for that particular fibre and are given ± S.E.M. where S.E.M. is larger than the symbol. The continuous line represents the linear regression to the data points (relative area = 1·01t − 27·4%, where t is the loading time; r = 0·966).

follows that the SR Ca^{2+} content is linearly related to the area under the caffeine-induced force responses for given experimental conditions. Separate experiments where the [EGTA] in solutions at the time of the caffeine application was reduced from 0·5 to 0·1 mM, have shown that for our loading conditions the SR begins loading Ca^{2+} with negligible delay after transferring the preparation to the loading solution. Therefore, in order to obtain direct proportionality between areas under the caffeine-induced force responses and the SR Ca^{2+} content, it is necessary to move the origin of the graph in Fig. 1 to the point where the line intercepts the y-axis. In practical terms it means that areas that are corrected by adding a constant equivalent to the y-axis intercept taken with opposite sign will be directly proportional to the SR Ca^{2+} content. Therefore the Ca^{2+} retention index can be obtained from the ratio of the respective corrected areas. The value of the y-axis intercept in Fig. 1 was −27·4% of the area corresponding to standard loading (120 s).

We have also determined the y-intercepts (expressed as a percentage of the respective areas corresponding to 120 s loading) for the following conditions used in this study: mechanically skinned toad fibres (−25·8%); mechanically skinned rat fibres after 10 min exposure to 10 μg ml^{-1} saponin (−23·2%); mechanically skinned rat and toad fibres after exposure to RR (−21·2 and −45·9%, respectively); mechanically skinned rat and toad fibres after exposure to saponin and after exposure to RR (−25·4 and −23·0%, respectively). For the mechanically skinned toad fibres after saponin treatment (30 min exposure to 150 μg ml^{-1} saponin) the standard loading time was 240 s due to a much reduced area under the caffeine-induced force response for 120 s loading and the value of the y-axis intercept was −22·4%. The y-intercepts were used to correct the relevant areas and to calculate the respective retention indices (Figs 8 and 9).

Analysis of results

In the text, mean values ± standard error of the mean are given; n is the number of fibres. One-way ANOVA followed by Student's t test was used to determine statistical significance (P) as

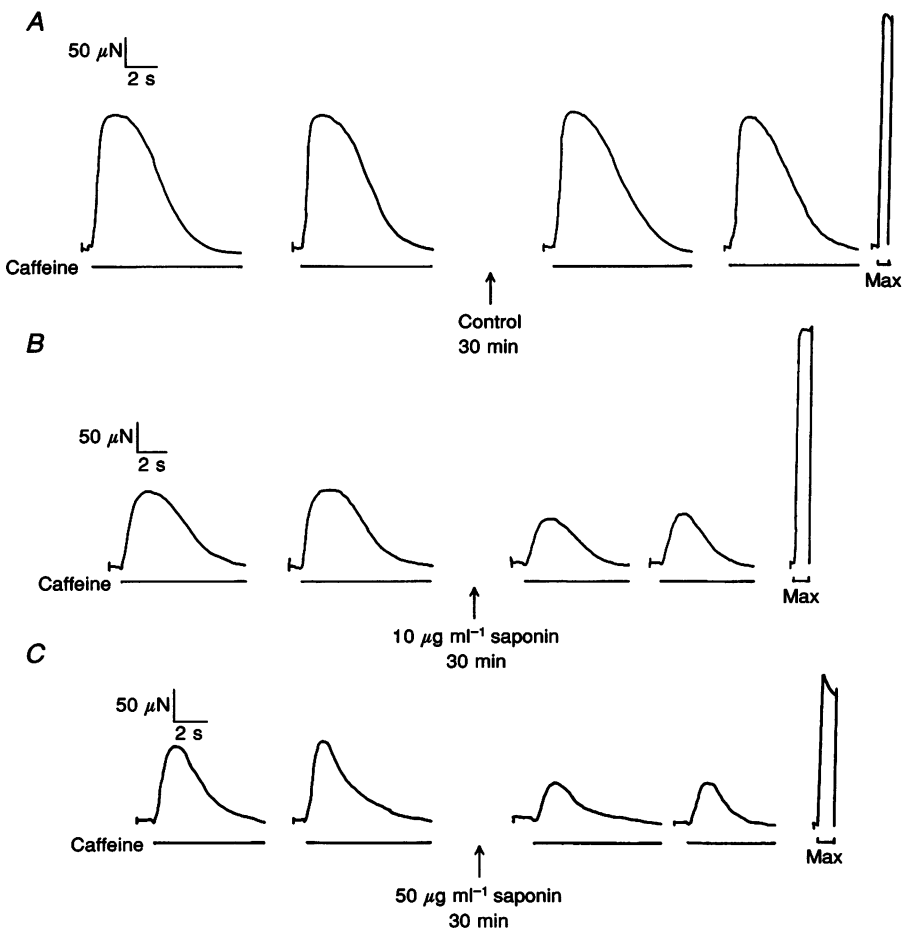

Figure 2. Effect of saponin treatment on SR Ca^{2+} accumulation in mechanically skinned muscle fibres of the rat

Paired caffeine-induced force responses before and after 30 min exposure to high-K^+ solution containing no saponin (control; *A*), 10 μg ml^{-1} saponin (*B*) and 50 μg ml^{-1} saponin (*C*). Ca^{2+} loading and releasing conditions were identical before and after the 30 min exposure to the high-K^+ solution with or without saponin. The area under the caffeine-induced response was the area bounded by the caffeine-induced force response and the resting force in the Ca^{2+}-release solution. Last trace in each panel represents the response of the fibre to maximum Ca^{2+}-activation solution (Max; time scale, 30 s). Note the high level of reproducibility between consecutive caffeine-induced force responses. The open lines under under each trace indicate the time when the preparation was exposed to caffeine. Fibre dimensions: *A*, $L = 1{\cdot}9$ mm, $D = 31$ μm; *B*, $L = 1{\cdot}9$ mm, $D = 38$ μm; and *C*, $L = 1{\cdot}1$ mm, $D = 24$ μm.

appropriate. GraphPad software (Prism, San Diego, CA, USA) was used to fit data to theoretical curves.

RESULTS

These experiments were designed to determine the concentration of saponin at which the ability of the SR to load Ca^{2+} becomes affected in muscle of different species. For this purpose mechanically skinned fibres were loaded submaximally at pCa 6·7 with Ca^{2+} under carefully defined conditions to facilitate detection of changes in the ability of the SR to load Ca^{2+}. The Ca^{2+} loading conditions (see Methods) were held constant for each preparation before and after 30 min exposure to high-K^+ solutions with different saponin concentrations. Control experiments were also performed with preparations exposed to high-K^+ solution for 30 min in the absence of saponin.

Effect of saponin treatment on the Ca^{2+} loading ability of mechanically skinned muscle fibres of the rat

Submaximal loading of the SR with Ca^{2+} under our standard conditions produced caffeine-induced force responses which were highly reproducible (see Fig. 2*A–C*). The SR Ca^{2+} loading ability of rat fibres did not change after 30 min exposure to high-K^+ solution with no saponin present (control; relative response, 0·994 ± 0·083; $n = 5$; see Fig. 2*A*).

However, the ability of rat SR to load Ca^{2+} was significantly decreased by treatment with even the lowest concentration

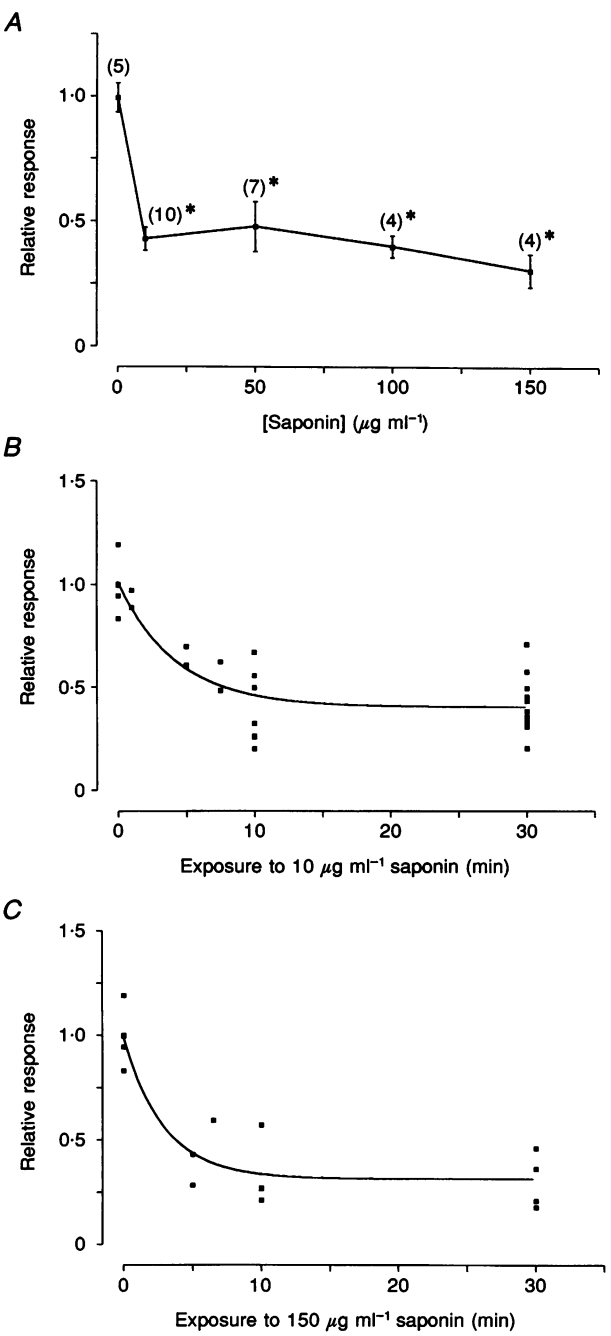

Figure 3. Decrease of Ca^{2+} loading of rat EDL mechanically skinned muscle fibres following exposure to saponin

A, summary of results obtained with 30 rat EDL skinned fibres showing the decrease in the SR Ca^{2+} loading ability (relative to pretreatment level in same fibre – see Methods) after exposure to different concentrations of saponin for 30 min (one-way ANOVA; $P < 0·0001$). Asterisks indicate significantly different results from controls (i.e. 30 min period with no saponin; $P < 0·01$; values of n given in parentheses). *B* and *C*, time course of the decrease of SR Ca^{2+} loading in rat EDL skinned fibres following treatment with 10 and 150 μg ml^{-1} saponin, respectively (relative response – see Methods). Results from 20 (*B*) and 11 (*C*) preparations; at 5, 10 and 30 min exposure to saponin there was overlay between individual data points in *B* ($n = 3$, 7 and 10 for 5, 10 and 30 min exposure, respectively). The continuous lines in *B* and *C* are exponential functions of type $y = A\exp\beta t + c$ which best fitted the data points. For *B*: $A = 0·60$, $\beta = -0·24$ min^{-1}, $c = 0·40$ and $r = 0·87$; for *C*: $A = 0·67$, $\beta = -0·34$ min^{-1}, $c = 0·32$ and $r = 0·92$. The data points at time 0 correspond to control responses when the preparations were exposed to high-K^+ solution without saponin for 30 min.

of saponin used in this study (10 μg ml^{-1}). The area under the caffeine-induced force response following treatment with 10 μg ml^{-1} saponin decreased by 57·2 ± 4·6% ($n = 10$) compared with control. Interestingly, increasing the saponin concentration from 10 to 50, 100 or 150 μg ml^{-1} did not further reduce the areas under the caffeine-induced response (reductions of the caffeine-induced force responses by 52·3 ± 7·0% ($n = 7$), 60·0 ± 9·2% ($n = 4$) and 69·6 ± 6·6% ($n = 4$) after exposure to 50, 100 and 150 μg ml^{-1} saponin, respectively, compared with controls in the absence of saponin; Figs 2 and 3*A*). It is important to note that there was no significant decrease in sensitivity to Ca^{2+} of the

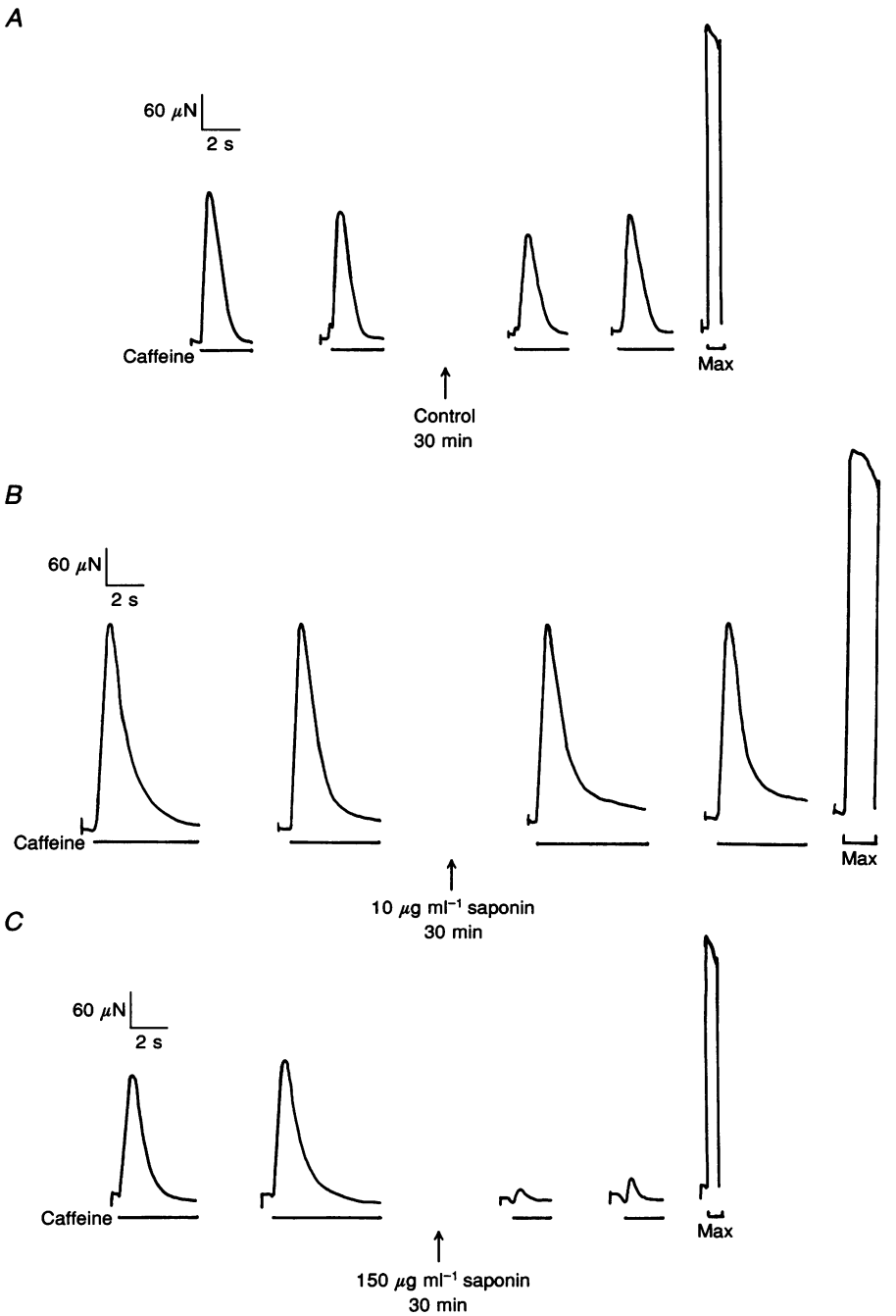

Figure 4. Effect of saponin treatment on SR Ca^{2+} loading in mechanically skinned muscle fibres from toad

Toad skinned fibres were loaded with Ca^{2+} at pCa 6·7 (loading solution) for 2 min and then the SR Ca^{2+} was released with caffeine in Ca^{2+} release solution (see Methods), before and after 30 min exposure to high-K^+ solution containing no saponin (control; *A*), 10 μg ml^{-1} saponin (*B*) and 150 μg ml^{-1} saponin (*C*). Last trace in each panel represents the response to maximum Ca^{2+}-activation solution (Max; time scale, 30 s). The open line under each trace indicates the time when the preparation was exposed to caffeine. Fibre dimensions: *A*, $L = 2$ mm, $D = 38$ μm; *B*, $L = 1·8$ mm, $D = 56$ μm; and *C*, $L = 1·2$ mm, $D = 38$ μm.

contractile apparatus as indicated by a series of control experiments in which the preparations were submaximally activated before and after 30 min treatment with 100 μg ml^{-1} saponin ($n = 4$; t test, $P > 0{\cdot}5$). Therefore, the decrease in the caffeine-induced responses after the saponin treatment must indicate a decrease in the SR Ca^{2+} content.

Since exposure to saponin was for 30 min in the above experiment, it was also important to find out whether the duration of exposure to saponin had an effect on the SR ability to load Ca^{2+} in mammalian fibres particularly at low saponin concentrations. For this purpose the duration of the exposure of preparations to 10 and 150 μg ml^{-1} saponin was varied between 1 and 30 min. As shown in Fig. 3*B* and *C*, the Ca^{2+} loading ability of the SR decreased exponentially with exposure time with rate constants of 0·24 and 0·34 min^{-1}, respectively, and approached a steady level for exposures of 10 min or more, indicating that prolonged exposure to saponin did not gradually decrease the ability of the SR to load Ca^{2+} as one would expect if saponin caused progressive perforation of the SR membrane.

Effect of saponin treatment on SR Ca^{2+} loading in mechanically skinned muscle fibres of the toad

Figure 4 shows representative responses with toad muscle preparations exposed to high-K^+ solution without saponin (control) or with 10 μg ml^{-1} or 150 μg ml^{-1} saponin. The relative response after 30 min exposure to high-K^+ solution with no saponin was 1·03 ± 0·17 ($n = 5$), which indicates that SR Ca^{2+} loading was not affected by time in high-K^+ solution (Fig. 4*A*). Exposure to 10 or 100 μg ml^{-1} saponin caused no statistically significant decrease in the areas under the caffeine-induced responses. However, increasing saponin concentration to 150 μg ml^{-1} produced a marked, statistically significant decrease in the caffeine-induced force response (Fig. 4*C*). The summary of results obtained with twenty toad fibres is shown in Fig. 5*A*. As was the case with rat skinned fibres, there was no significant decrease in sensitivity to Ca^{2+} of the contractile apparatus as indicated by control experiments similar to that performed on rat fibres ($n = 5$; t test, $P = 0{\cdot}49$). Therefore, the decrease in caffeine-induced responses after saponin treatment could only be attributed to a decrease in the SR Ca^{2+} content. As was the case with the rat fibres, the ability of the toad fibres to load Ca^{2+} decreased exponentially with exposure to 150 μg ml^{-1} saponin with a rate constant of 0·22 min^{-1} and approached a steady level for exposures of 10 min or more (Fig. 5*B*).

Effect of saponin treatment on SR Ca^{2+} loading ability in mechanically skinned muscle fibres of the yabby

The caffeine-induced responses in yabby fibres were not significantly changed after 30 min in the high-K^+ solution (0·829 ± 0·105; $n = 3$; t test, $P > 0{\cdot}05$) (see Fig. 6*A*). Exposure to 10 μg ml^{-1} saponin for 30 min, however, caused

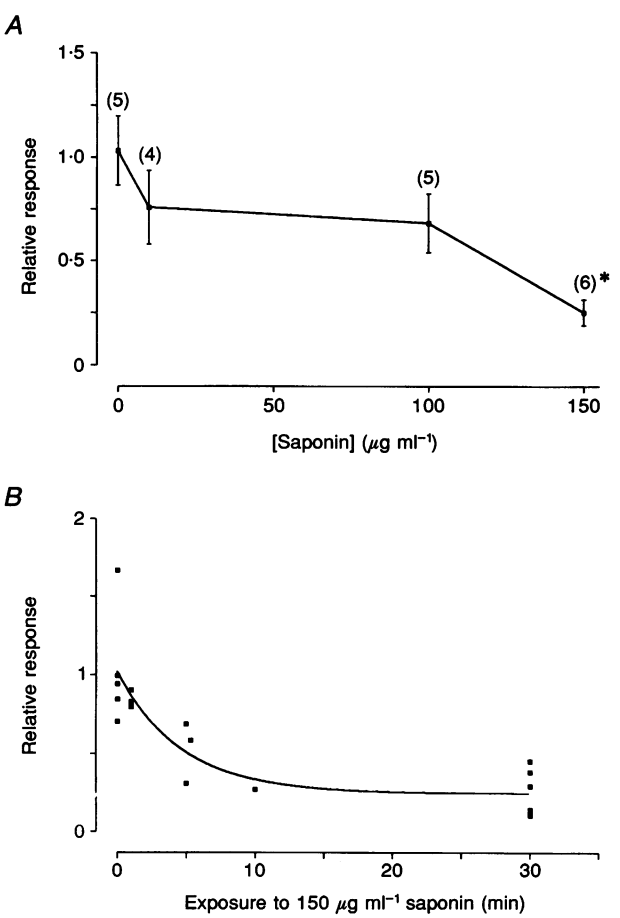

Figure 5. Effect of saponin on SR Ca^{2+} loading of toad mechanically skinned muscle fibres

A, summary of results obtained from 20 toad iliofibularis skinned fibres which shows that the SR Ca^{2+} loading ability was not significantly affected when the skinned fibres were treated with saponin at concentrations $\leqslant$ 100 μg ml^{-1} for 30 min but that it decreased markedly compared with controls when the saponin concentration was increased to 150 μg ml^{-1} (one-way ANOVA; $P = 0{\cdot}006$). Only the relative response after 30 min exposure to 150 μg ml^{-1} saponin is significantly different from the control (unpaired t test, $P = 0{\cdot}002$). *B*, time course of the decrease in SR Ca^{2+} loading in toad iliofibularis skinned fibres following treatment with 150 μg ml^{-1} saponin. The data points (from 14 preparations) best fitted the exponential function $y = A\exp\beta t + c$, where $A = 0{\cdot}78$, $\beta = -0{\cdot}22$ min^{-1}, $c = 0{\cdot}25$; $r = 0{\cdot}85$).

complete loss of the caffeine-induced response after SR Ca^{2+} loading under our standard conditions in each of three preparations investigated (see Fig. 6*B*), which could not be explained by a relatively small use dependent decrease in Ca^{2+} sensitivity observed in four control fibres following exposure to 10 μg ml^{-1} saponin for 30 min. Increasing the loading time and [Ca^{2+}] in the loading solution to 5 min and pCa 6·1, respectively, led to very small caffeine-induced responses indicating that the ability of the SR to load Ca^{2+} after exposure to very low saponin concentrations was severely impaired.

The mechanism of saponin-induced inhibition of SR Ca^{2+} loading ability

As mentioned earlier, it is accepted that saponin can perforate membranes containing cholesterol. Since the SR membranes of mammalian muscles contain very little cholesterol (Meissner & Fleischer, 1971) it was expected that at low concentrations, saponin should not have affected the SR Ca^{2+} loading ability. However, from results shown in Figs 2 and 3 it is clear that low saponin concentrations had a very marked effect on mammalian SR after a short period of exposure. It is therefore possible that saponin may also have a more subtle effect on the SR by affecting the Ca^{2+} release channels. One possibility was that saponin perforated the sealed transverse tubular system (t-system) in mechanically skinned fibres and caused chronic depolarization of the t-system and inactivation of the voltage sensors, which could have affected the open probability of the Ca^{2+} release channels. In order to test this hypothesis a series of experiments were performed in Na^+-based solutions in which the t-system was chronically depolarized. Treatment with 50 μg ml^{-1} saponin for 30 min caused the same decrease in ability of the SR to load Ca^{2+} in the Na^+ solutions as in the K^+ solutions in rat fibres (relative responses: 0·395 ± 0·140 ($n = 5$) and 0·477 ± 0·070 ($n = 7$) for Na^+ and K^+ solutions, respectively; t test, $P > 0·1$) clearly demonstrating that the saponin-induced leak was not associated with the inactivation of the voltage sensors following the treatment with saponin. Another possibility was that the Ca^{2+} release channels were directly affected by the saponin treatment. In order to test this, the Ca^{2+} release channel blocker Ruthenium Red (RR; Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1988) was used to determine if the inhibitory action of saponin treatment on the SR ability to load Ca^{2+} was caused by loss of SR Ca^{2+} through non-specific pores, which should not be affected by RR, or through the Ca^{2+} release channel, which should be blocked by RR.

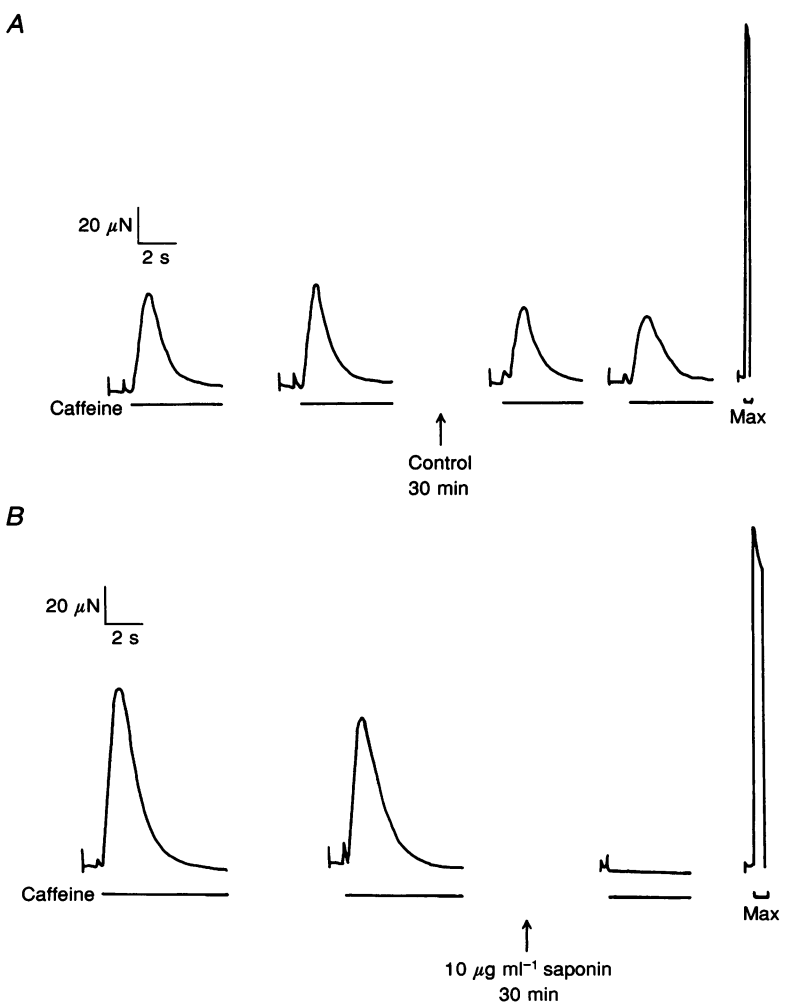

Figure 6. Effect of saponin treatment on SR Ca^{2+} loading in mechanically skinned muscle fibres of the yabby

Yabby skinned fibres were loaded with Ca^{2+} and Ca^{2+} was released with caffeine in Ca^{2+} release solution as indicated in the Methods. Paired caffeine-induced force responses before and after exposure to high-K^+ solution with no saponin (control, *A*) and 10 μg ml^{-1} saponin (*B*). The last trace response for each panel represents the force reponse to maximum Ca^{2+}-activation solution (Max; time scale, 30 s). The open line under each trace indicates the time when the fibre was exposed to caffeine. Fibre dimensions: *A*, $L = 1·9$ mm, $D = 50$ μm; and *B*, $L = 2·5$ mm, $D = 38$ μm.

In the presence of 50 μM RR, 30 mM caffeine was not able to release Ca^{2+} from the SR in any of the three rat or the four toad skinned muscle fibres investigated even when the SR was heavily loaded with Ca^{2+} and [Mg^{2+}] was lowered to 50 μM. The presence of large amounts of Ca^{2+} in the SR was indicated by the large and prolonged force responses obtained when the SR was subsequently lysed in the presence of the non-ionic detergent Triton X-100 (Fig. 7). However, after short exposure (1 min) to lower RR concentrations (6 μM), the SR Ca^{2+} release channels did not

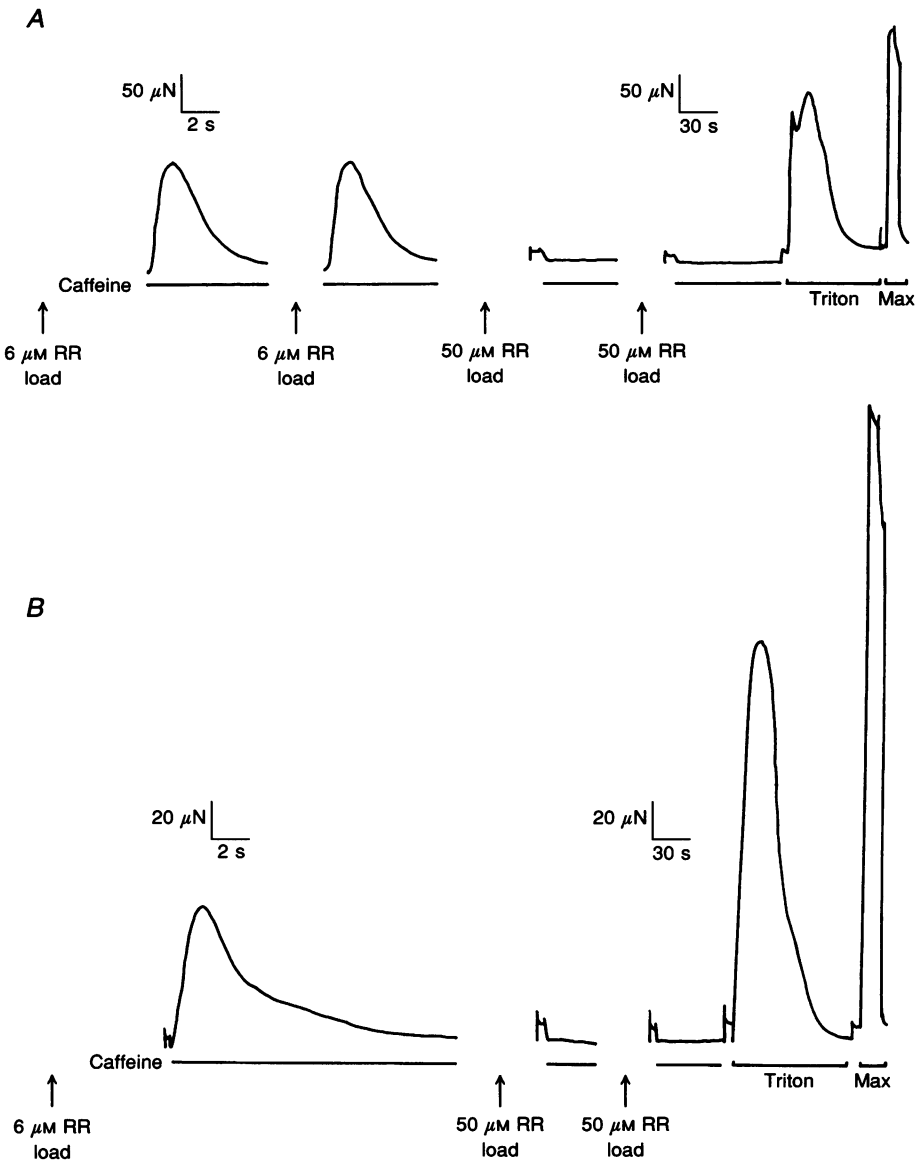

Figure 7. Effect of RR on caffeine-induced SR Ca^{2+} release

A, 30 mM caffeine in Ca^{2+} release solution could release SR Ca^{2+} from toad fibres after being loaded with Ca^{2+} and pre-equilibrated in high-K^+ solution in the presence of 6 μM RR (1st and 2nd responses; RR was not present in caffeine solutions). When SR Ca^{2+} loading and 30 s pre-equilibration were performed in the presence of 50 μM RR, 30 mM caffeine in Ca^{2+} release solution could not induce the release of SR Ca^{2+}. Lysing all membraneous compartments with Triton X-100 in the presence of 0·1 mM EGTA produced a near maximal force response indicating that there was a large amount of Ca^{2+} in the SR. *B*, rat fibre loaded with Ca^{2+}, pre-equilibrated for 30 s in high-K^+ solution in the presence of 6 μM RR and then exposed to Ca^{2+} release solution. Caffeine could not elicit a transient force response when the SR was loaded with Ca^{2+} in the presence of 50 μM RR and then pre-equilibrated in high-K^+ solution with 50 μM RR (2nd and 3rd exposure to caffeine). Disruption of the SR membranes with Triton X-100 produced a large force response indicating that the SR contained a large amount of Ca^{2+}. Maximum Ca^{2+}-activated force is shown at the right of each trace. The 30 s time scale refers to Triton and Max responses only. The open lines under each trace in *A* and *B* indicate exposure to Ca^{2+} release solution. Fibre dimensions: *A*, $D = 31$ μm, $L = 1{\cdot}9$ mm; and *B*, $D = 31$ μm, $L = 1{\cdot}4$ mm.

appear to be completely blocked as caffeine was able to release Ca^{2+} from the SR and induce large force responses in both rat and toad fibres (Fig. 7). Therefore, we used 6 μM RR to partially block the Ca^{2+} release channels of both toad and rat skinned fibres so that caffeine could still induce Ca^{2+} release from the SR and test whether RR did reverse the effect of saponin on the Ca^{2+} permeability of the SR. It is important to point out that there is evidence that 10 μM RR does not affect the SR Ca^{2+} pump under our conditions (Kabbara & Stephenson, 1994).

Yabby preparations were not sensitive to 6 μM RR and if anything the responses were smaller (data not shown). This is consistent with reports that caffeine-induced responses in skinned crayfish fibres are not inhibited by 30 μM RR (Zacharová, Uhrík, Hencek, Lipskaja & Pavelková, 1990) and that even high RR concentrations (1 mM) could only moderately inhibit Ca^{2+}-activated Ca^{2+} efflux from crayfish SR vesicles (Seok, Xu, Kramarcy, Sealock & Meissner, 1992).

The Ca^{2+} permeability of the SR was determined by pre-equilibrating the fibre in high-K^+ solutions for 30 and 120 s after loading Ca^{2+} to the same initial level and then releasing Ca^{2+} in Ca^+ release solution (see Methods and Fig. 8). To obtain a measure of the relative SR Ca^{2+} permeability the SR Ca^{2+} loading curves calculated for all loading conditions were used to correct the areas under the caffeine-induced force responses after 120 and 30 s pre-equilibration in the leak solutions ensured that the ratio between the corrected areas was proportional to the SR Ca^{2+} content (see Methods). With these corrected values a Ca^{2+} retention index ($[Ca^{2+}]_{SR,120s}/[Ca^{2+}]_{SR,30s}$) was calculated which is directly dependent on the SR permeability to Ca^{2+} (see Methods, eqn (3)). Exposure of mechanically skinned fibres from the rat to RR did not affect the Ca^{2+} retention index ($0{\cdot}75 \pm 0{\cdot}08$, $n = 4$, and $0{\cdot}76 \pm 0{\cdot}06$, $n = 4$, respectively; t test, $P > 0{\cdot}5$) indicating that the SR Ca^{2+} loss is not affected by partially blocking the SR Ca^{2+} release channels.

After the rat fibres were exposed to 10 μg ml^{-1} saponin for 10 min (in the absence of RR), the loss of SR Ca^{2+} increased, as shown by the statistically significant decrease in the Ca^{2+} retention index from $0{\cdot}75 \pm 0{\cdot}08$ to $0{\cdot}48 \pm 0{\cdot}06$ ($n = 6$; t test, $P = 0{\cdot}01$). This corresponds to an increase in the SR permeability for Ca^{2+}, P_{Ca}, by a factor of 2·6 ($\ln 0{\cdot}48/\ln 0{\cdot}75$; eqn (3)) and provides strong further evidence that 10 μg ml^{-1} saponin affects the SR, considering that the retention index should be independent of the kinetics of caffeine-induced Ca^{2+} release (see Methods). The application of 6 μM RR for 1 min after the saponin treatment significantly increased the Ca^{2+} retention index to $0{\cdot}68 \pm 0{\cdot}05$ ($n = 6$; t test, $P = 0{\cdot}03$), which was not significantly different from that before exposure to saponin (t test, $P > 0{\cdot}1$). This indicates that the marked increase in SR permeability to Ca^{2+} was almost completely reversed by RR suggesting that saponin made the SR Ca^{2+} release channels more leaky.

Toad skinned fibres displayed similar behaviour to the rat fibres. Before saponin treatment the Ca^{2+} retention index was similar in control ($0{\cdot}76 \pm 0{\cdot}08$; $n = 6$) and after exposure to 6 μM RR for 1 min ($0{\cdot}83 \pm 0{\cdot}02$; $n = 4$; t test, $P = 0{\cdot}45$). Treatment for 30 min with 150 μg ml^{-1} saponin significantly decreased (t test, $P < 0{\cdot}01$) the Ca^{2+} retention

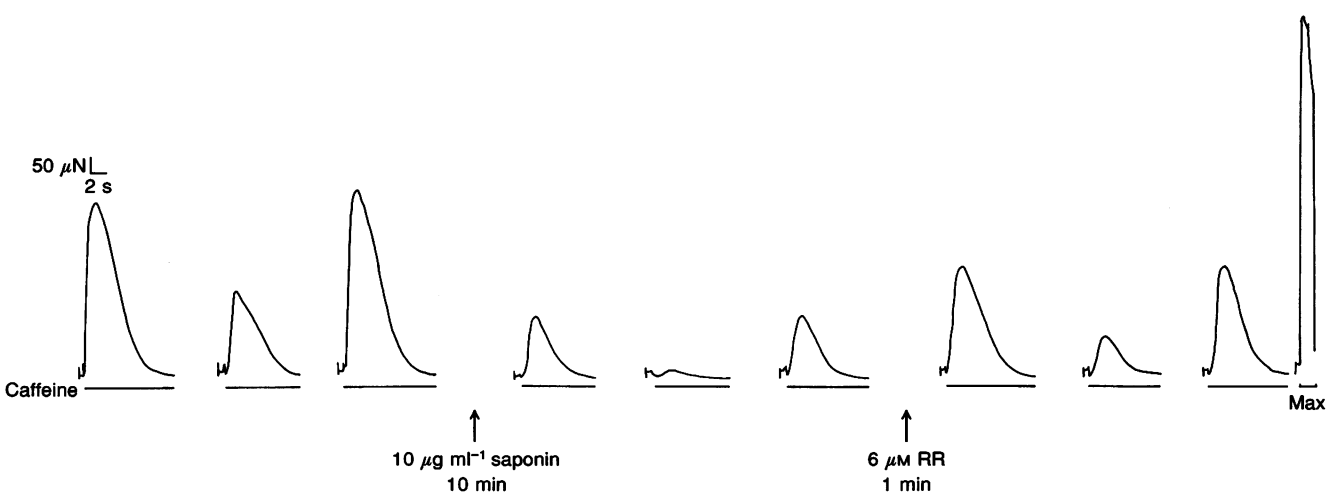

Figure 8. Representative result with a skinned muscle fibre of the rat showing that the saponin-induced SR Ca^{2+} leak could be reversed by RR

Each group of traces represent caffeine-induced responses from the preparation after 30 s, 120 s and again 30 s equilibration in high-K^+ solution following SR Ca^{2+} loading to the same level. The first three traces are before exposure to saponin, the second three traces are after exposure to 10 μg ml^{-1} saponin for 10 min and the last group of three traces are after 1 min exposure to 6 μM RR in high-K^+ solution. The last trace represents the response to maximum Ca^{2+}-activation solution (Max; time scale, 30 s). The open lines under each trace indicate exposure to caffeine. Fibre dimensions: $L = 1{\cdot}1$ mm, $D = 38$ μm.

index to 0·40 ± 0·04 ($n = 4$) and this corresponds to an increase in P_{Ca} by a factor of 3·3 (ln 0·40/ln 0·76). As is the case with rat fibres, the significant decrease in the Ca^{2+} retention index conclusively shows that 150 μg ml^{-1} saponin affects the SR in toad muscle. Treatment with 6 μM RR for 1 min caused a significant increase in the Ca^{2+} retention index (t test, $P < 0·01$) to a value similar to that before saponin treatment (0·68 ± 0·07; $n = 5$).

The increased Ca^{2+} permeability induced by saponin treatment that could be blocked by RR in both rat and toad fibres indicates that the Ca^{2+} release channels had been made more permeant to SR Ca^{2+} by saponin treatment (Fig. 9). Based on eqn (3), the corresponding rate constants (αP_{Ca}) for the passive efflux (in the absence of RR) of SR Ca^{2+} for rat and toad fibres are 0·004 ± 0·001 s^{-1} and 0·003 ± 0·001 s^{-1}, respectively.

DISCUSSION

Saponin effects on skeletal muscle SR

Results obtained in this study clearly demonstrate that skeletal muscle SR of eutherian mammals, anurans and decapod crustaceans respond differently to saponin treatment. Saponin was applied to mechanically skinned fibre preparations and the SR was loaded submaximally with Ca^{2+}, which allowed easy identification of any changes in the SR Ca^{2+} loading ability after saponin treatment.

We could find no evidence that exposure of SR of anuran skeletal muscle fibres to saponin concentrations $\leqslant$ 100 μg ml^{-1} for 30 min affected SR Ca^{2+} loading properties in any significant way. This further supports the conclusion reached by Endo & Iino (1980) and Fano *et al.* (1989) that saponin is an appropriate agent to chemically permeabilize the surface membrane and investigate the SR properties of anurans. Interestingly, we have found that even low concentrations of saponin have a significant effect on the Ca^{2+} loading ability of the SR of crustacean and mammalian skeletal muscle. Yabby SR was most sensitive to saponin treatment: the Ca^{2+} loading ability was completely abolished by exposure to 10 μg ml^{-1} saponin under our loading conditions (Fig. 6). In rat, the Ca^{2+} loading ability of the SR was reduced markedly after treatment with 10–150 μg ml^{-1} saponin (Figs 2 and 3). These results not only highlight problems with using saponin to skin mammalian and crustacean skeletal muscle fibres but also raise the possibility that SR from other muscle types is sensitive to saponin treatment.

An important observation was that the Ca^{2+} loading ability of rat fibres was decreased to the same steady level after 30 min treatment with all saponin concentrations tested here (Fig. 3*A*). Furthermore, concentrations of 10 and 150 μg ml^{-1} saponin were found to reduce the Ca^{2+} loading ability of rat fibres to this steady level with rate constants of 0·24 and 0·34 min^{-1}, respectively (Fig. 3*B* and *C*). Interestingly, Donaldson (1985) showed that exposure of rabbit muscle fibres to 500 μg ml^{-1} saponin for 1 min reduced the ability of the SR to load Ca^{2+} by about 20% (Fig. 5 in Donaldson (1985)), which is similar to the reduction observed in our experiments when rat fibres were exposed to only 10 or 150 μg ml^{-1} saponin for 1 min.

The partial inhibition of SR Ca^{2+} loading ability in rat skeletal muscle induced by saponin does not appear to be

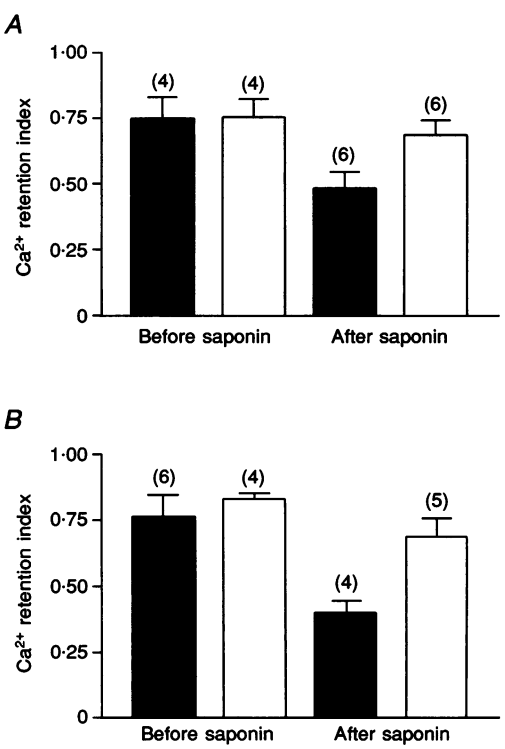

Figure 9. The effect of saponin and RR on the Ca^{2+} retention index in rat and toad muscle fibres

Summary of results showing the Ca^{2+} retention index, $[Ca^{2+}]_{SR,120s}/[Ca^{2+}]_{SR,30s}$, in rat and toad fibres in the absence (■) and presence (□) of 6 μM RR before and after exposure to saponin (one-way ANOVA; $P < 0·01$ for both *A* and *B*). *A*, rat fibres did not show a significant difference in Ca^{2+} retention index after exposure to 6 μM RR before treatment with saponin. After exposure to 10 μg ml^{-1} saponin for 10 min, the Ca^{2+} retention index significantly decreased and this could be restored after RR treatment (see protocol in Fig. 8). *B*, toad fibres also did not show a significant difference in Ca^{2+} retention index after exposure to 6 μM RR prior to saponin treatment and the significant decrease in the retention index caused by exposure to 150 μg ml^{-1} saponin for 30 min could be restored after treatment with 6 μM RR (see also text).

dose dependent or to depend on whether the t-system was chronically depolarized (and consequently the voltage sensors inactivated) or not. The reduction of the SR Ca^{2+} loading ability to the same steady level by 10–100 $\mu g\ ml^{-1}$ saponin is inconsistent with the idea that saponin perforates the SR membrane in the same way as it does the sarcolemma. Indeed, the concentration of cholesterol in the SR membrane is very low (< 2% of the total SR lipid (Meissner & Fleischer, 1971)) and the Ca^{2+} release channel blocker RR was able to block the saponin-induced Ca^{2+} leak through the SR membrane (Figs 7 and 8). Toad SR displayed resistance to saponin concentrations below 100 $\mu g\ ml^{-1}$ but an increased SR Ca^{2+} permeability was observed upon treatment with 150 $\mu g\ ml^{-1}$ saponin. The increased SR Ca^{2+} leak in toad fibres could also be significantly reduced with RR. The blocking action of RR must therefore have been exerted via the Ca^{2+} release channels which had their properties altered by saponin in an indirect or direct fashion. For example, the removal of the small amount of cholesterol molecules from the SR membrane may have increased the fluidity of the lipid bilayer and thus could have caused an increase in the open probability of the Ca^{2+} release channel. Alternatively, saponin may have altered in a more direct way the tertiary structure of the Ca^{2+} release channel. The precise action of saponin on the Ca^{2+} release channel is not known, but it may be explained by modification of either the activation or inhibition properties of the Ca^{2+} release channel.

The mechanism responsible for the loss of Ca^{2+} loading ability in yabby fibres after exposure to saponin could not be investigated using RR because RR was ineffective in blocking the Ca^{2+} release channel (Zacharová *et al.* 1990; and Seok *et al.* 1992). Therefore it is difficult to distinguish between the possibility (1) that the yabby SR has a relatively high cholesterol content and is attacked by saponin and (2) that saponin has a more direct effect on the Ca^{2+} release channel, similar to that occurring in rat and toad muscle.

One should bear in mind that when saponin is applied to an intact muscle fibre the SR would be exposed to saponin for shorter periods of time because of the delay in entering the cytosol. Therefore, it is still possible that low concentrations of saponin applied to intact muscle fibre bundles for short periods of time do not adversely affect the SR. Nevertheless, it must still be realized that mammalian and crustacean skeletal muscle SR is very sensitive to saponin when it is applied directly to these membranes and therefore periods of application of saponin to intact muscle fibres should be minimized to avoid adverse effects on SR properties.

Ca^{2+} efflux from the SR of skinned skeletal muscle fibres

We have shown that the apparent rate constant for Ca^{2+} efflux is similar in rat and toad fibres ($0{\cdot}004 \pm 0{\cdot}001$ and $0{\cdot}003 \pm 0{\cdot}001\ s^{-1}$, respectively) under our conditions. Assuming that the maximum rate of Ca^{2+} release is $150\ s^{-1}$ in rat at 22 °C, the same as measured in mouse at 16 °C (Hollingworth, Zhao & Baylor, 1996), the passive rate constant would be only 3×10^{-5} of that at the peak rate of Ca^{2+} release. This suggests that in skinned fibres the Ca^{2+} release channel is tightly closed whilst it is in the resting state. To compare the passive efflux rate with that observed in fragmented SR, a correction factor was calculated as follows: a factor of 1·3 was estimated to allow for the difference in surface area : volume ratio between intact terminal cisternae and heavy SR vesicles (Deamer & Baskin, 1969; Eisenberg & Kuda, 1975) and a factor of 4·06 was calculated from the ratio of total surface area of the cisternae to the junctional surface and assuming that 70% of the t-system is in contact with the cisternae (Peachy, 1965; Eisenberg & Kuda, 1975; Mobley & Eisenberg, 1975) to allow for the possibility that the heavy SR vesicles were made entirely from the junctional area of the cisternae. With an overall correction factor of 5·25 ($1{\cdot}3 \times 4{\cdot}06$) one would expect that the rate constant for SR vesicles to be $0{\cdot}026\ s^{-1}$ based on our results on skinned fibres. The passive rate constant measured by Meissner (1984) was about $0{\cdot}018\ s^{-1}$ for equivalent conditions (1·5 nmol Ca^{2+} (mg protein)$^{-1}$ s^{-1}/80 nmol Ca^{2+} (mg protein)$^{-1}$), which is well within the range for the corrected value for skinned muscle fibres.

It was also interesting to note from the Ca^{2+} leak experiments that addition of 6 μM RR did not cause a statistically significant increase in the Ca^{2+} retention index suggesting either that the residual Ca^{2+} leak from the SR at low $[Ca^{2+}]$ in the myoplasm does not take place through the Ca^{2+} release channel or that the Ca^{2+} release channels are able to flicker at a basal rate, as one would expect if RR binds to an inhibitory site rather than simply blocking the channel. Interestingly, 2 μM RR blocked E–C coupling in toad and rat fibres (Lamb & Stephenson, 1990*b*, 1991*b*) but we found here that 50 μM RR was necessary to block entirely the caffeine-induced opening of the channels (Fig. 7) indicating that depolarization-induced opening of the SR Ca^{2+} release channels is more sensitive to RR than caffeine-induced opening of the channels. This is similar to the previous observation that 10 mM Mg^{2+} could completely inhibit the depolarization-induced Ca^{2+} release in both toad and rat muscle fibres (Lamb & Stephenson, 1991*a*, 1994) in the absence of caffeine but not when caffeine was present (Lamb & Stephenson, 1991*b*). Therefore the suggestion is made that RR has a similar effect to Mg^{2+} by acting on an inhibitory site rather than blocking the channel.

Concluding remarks

Results obtained in this study clearly indicate that at relatively low concentrations, saponin does affect the SR Ca^{2+} release channel properties in mammalian and anuran skeletal muscle. The SR in decapod crustacean skeletal muscle was found to be particularly sensitive to saponin, which caused irreversible loss of ability to load Ca^{2+} at 10 $\mu g\ ml^{-1}$.

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